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(21) International Application Number: PCT/GB89/00965 (22) International Filing Date: 18 August 1989 (18.08.89) (30) Priority data: 8819836.1 20 August 1988 (20.08.88) GB (71) Applicant (for all designated States except US): INSTITUTE FOR ANIMAL HEALTH LIMITED [GB/GB]; Comp-ton, Nr Newbury, Berkshire RG16 0NN (GB). (72) Inventors; and (75) Inventors/Applicants (for US only) : BLACK, Donald, Norman [GB/GB]; BOSTOCK, Christopher, John [GB/GB]; GERSHON, Paul, David [GB/GB]; AFRC Institute for Animal Health Limited, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF (GB).		(74) Agent: BASSETT, Richard, Simon; Eric Potter & Clarkson, 14 Oxford Street, Nottingham NG1 5BP (GB). (81) Designated States: AT (European patent), AU, BE (European patent), BF (OAPI patent), BJ (OAPI patent), CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), FR (European patent), GA (OAPI patent), GB (European patent), IT (European patent), LU (European patent), ML (OAPI patent), MR (OAPI patent), NL (European patent), SE (European patent), SN (OAPI patent), TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limits for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: VACCINE (57) Abstract A heterologous coding sequence, for example for an antigen associated with a viral or other disease, is inserted in a non-essential region of the capripoxvirus genome, for example the TK (thymidine kinase) coding region or the open reading frames designated herein as CT3C, CT4 or Q2.		

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VACCINE

The present invention relates to vaccines in which a viral vector is used to express a heterologous gene.

Capripoxvirus causes sheep pox and goat pox, diseases which can kill over 50% of young animals in those areas, principally Africa and the Middle and Far East, where the disease is enzootic. Different strains of the virus show a preference for either sheep or goats but none have so far been found to be totally species-specific. Some strains also infect, and cause lumpy skin disease in, cattle but (apart from buffalo) capripox-viruses do not infect any other domestic animals. A vaccine has been developed which comprises an attenuated strain of the strain known as "the Kenya isolate" (Kitching, Rev. Sci. Tech. Off. Epiz., 1986, 5(2), 503-511).

It is known that the vaccinia virus genome can be altered by insertion of a heterologous gene from a pathogen, such that the product of that gene will be expressed in infected cells. Protection against diseases

associated with the pathogen has been induced in this way. A review of such work may be found in Mackett & Smith, J. Gen. Virol. 67. 2067-2082 (1986).

At a U.N. Food and Agriculture Organisation meeting in Madrid in October 1986, Mowat disclosed that capripoxvirus represented a possible alternative to vaccinia as a vector for foot and mouth disease vaccines. In the 1987 Annual Report of the Institute for Animal Health, Compton, U.K., (published April 1988) Black and Gershon disclosed that work was in progress on the use of capripoxvirus as a vector for heterologous antigens and that the thymidine kinase gene was a candidate insertion site.

We have now shown that capripoxvirus can indeed be used as a vector for heterologous genes and that there are several possible insertion sites for such genes.

One aspect of the invention provides a capripoxvirus capable of infecting and replicating in capripoxvirus-susceptible cells and carrying at least one heterologous gene encoding an antigen of a pathogen such that the gene will be expressed during transcription and translation from the capripoxvirus genome in the said cell.

Preferably, the heterologous gene is inserted in one of the following regions of the capripoxvirus genome:

- (a) the thymidine kinase gene region,
- (b) the CT3C region,
- (c) the C4 region, and
- (d) the Q2 region.

By "thymidine kinase gene region" we mean the genetic unit, including coding and non-coding sequences, for the expression of thymidine kinase.

By "CT3C region" and "CT4 region", we mean the respective regions of the capripox virus genome identified by these designations in the attached figures, or variations thereof. By "Q2 region", we mean the approximately 2.4 kb fragment bounded by the respective HindIII sites approximately 6.6 and 9.0 kb from the right-hand end of the KS-1 isolate genome shown in Figure 1 and identified thereon as Q2, and variations thereof. Such variations of any of these regions will include: minor polymorphic changes associated with different

strains of capripoxvirus; any other changes which might occur or be created in the nucleic acid of capripoxvirus but which do not alter the non-essential nature of the said region or the nature of the gene product encoded by that region; and any other nucleotide sequences which encode a gene product with substantially the same structure or function as the gene product of the said region or which correspond to the said region but are non-coding. In general, nucleotide sequences with at least 80% homology to the said regions are regarded as variations thereof. Preferably, the extent of homology is 85%, 90%, 95% or 99%.

By "antigen" we mean any structure, part thereof or mimic thereof associated with a pathogen and capable of giving rise to an immune response in a host when the immune system of that host is exposed to the antigen. Preferably, the immune response is a protective response. Thus, surface carbohydrates, glycoproteins and proteins of viruses and other pathogens are included in this definition, as are peptides which are homologous to part of such whole molecules, and peptides which mimic the tertiary structure of such molecules. In the case of antigens which are glycoproteins, the heterologous gene will encode the amino acid sequence of the glycoprotein

and/or one of more enzymes which cause appropriate glycosylation of that amino acid sequence. In the case of antigens which are carbohydrates, the heterologous gene or genes will encode one or more enzymes which catalyse the synthesis of such carbohydrates.

By selecting appropriate heterologous genes from other pathogens, it is possible to use the vectors of the invention in vaccines effective in preventing a wide variety of diseases in sheep, goats and cattle. The following is a non-exhaustive list of such diseases:

<u>Viral disease</u>	<u>Host</u>
Rotavirus infection	Sheep, cattle
* Bluetongue	Sheep, goats, cattle
+ Ephemeral fever	Cattle
* Foot-and-mouth disease	Sheep, cattle, goats wild ruminants
Infectious bovine rhinotracheitis	Cattle
Infectious pustular vulvovaginitis	Cattle
Malignant catarrh	Sheep, cattle
+ Mucosal disease/bovine viral diarrhoea	Cattle
+ Border disease	Sheep
+ Rabies	Sheep, cattle,

	goats, wild ruminants
Rift Valley fever	Sheep, Cattle, goats,
	wild ruminants
* Rinderpest	Cattle, goats
* Peste des petits ruminants	Sheep, goats
+ Vesicular stomatitis	Sheep, goats, cattle
Nairobi Sheep disease	Sheep
Scrapie	Sheep
+ Lentivirus infection	Sheep, goats
Akabane	Cattle
<u>Rickettsial disease</u>	<u>Host</u>
Anaplasmosis	Cattle
Bovine petechial fever	Cattle
Heartwater	Cattle
Jembrana disease	Cattle
Enzootic abortion	Sheep
<u>Bacterial disease</u>	<u>Host</u>
Anthrax	Sheep, goats, cattle,
	wild ruminants, buffalo
Brucella abortus infection	Buffalo, cattle
Brucella melitensis infection	Cattle, sheep, goats,
	buffalo
Brucella ovis infection	Sheep

Clostridium septicum infection -

blackleg)	
malignant oedema)	Sheep, cattle, goats,
braxy)	buffalo

Clostridium chauvoei infection -

blackquarter		Sheep, cattle, goats,
		buffalo

Clostridium novyi (type A)

infection - Big head	Sheep
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Clostridium perfringens Sheep, cattle, goats,

infection	buffalo
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Clostridium novyi (type B)

infection - Black disease	Sheep, cattle, goats
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Clostridium novyi (type D)

infection - Bacillary Haemo-	
globinuria	Cattle, sheep, goats

Clostridium perfringens (type B)

infection - Lamb dysentery	Sheep
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Clostridium perfringens (type C)

infection	Sheep, cattle
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Clostridium perfringens (type D)

infection- Pulpy kidney disease	Sheep, goats
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Clostridium tetani infection - Sheep, goats, cattle,

Tetanus	wild ruminants
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Clostridium botulinum infection -

Botulism	Sheep, goats, cattle, wide ruminants
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Mycoplasma agalactia infection -

Contagious agalactin	Sheep, goats
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Mycoplasma mycoides infection -

Contagious bovine pleuro- pneumonia	Buffalo, cattle, wild ruminants
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Contagious caprine pleuro- pneumonia	Sheep, goats
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Pasteurella multocida infection -

Haemorrhagic septiceamia	Cattle, buffalo
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Mycobacterium johnei infection -

Johne's disease	Cattle, sheep, goats, buffalo
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<i>Leptospira</i> sps. infections	Cattle, sheep, goats, buffalo
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<i>Salmonella</i> sps. infections	Cattle, sheep, goats, buffalo
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<i>Mycobacterium tuberculosis</i> infection	Cattle, sheep, goats, buffalo
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<i>Pasteurella</i> sps. infection	Sheep, goats
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Protozoal disease

Babesia sps. infections	Cattle, buffalo, sheep, goats, wild ruminants
+ Theileria sps. infections	Cattle, buffalo, sheep, goats, wild ruminants
+ Toxoplasma gondii infection	Sheep
+ Trypanosoma sps. infections	Cattle, buffalo, sheep, goats, wild ruminants

HostHelminth disease

Bunostomum sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Chabertia sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Taenia sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Trichuris sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Microcoelium infection	Sheep, goats, cattle, buffalo, wild ruminants
Dictyocaulus sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Echinococcus sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Fasciola sps. infection	Sheep, goats, cattle, buffalo, wild ruminants

Host

Haemonchus sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Oesophagostomum sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Onchocerca sps. infection	Cattle, buffalo
Ostertagia sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Paramphistoma sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Schistosoma sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Stephanofilaria sps. infection	Cattle, buffalo
Strongylus sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Thelazia sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Trichostrongylus sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Cooperia sps. infection	Sheep, goats, cattle, buffalo, wild ruminants
Nematodirus sps. infection	Sheep, goats, cattle, buffalo, wild ruminants

Genes have already been isolated for antigens in those viruses marked with an asterisk or a cross.

The recombinant viruses of the invention may be prepared by (i) constructing a DNA construct comprising the heterologous gene and two sequences which flank the heterologous gene and which are homologous to respective parts of the capripoxvirus DNA, such that recombination of the construct with capripoxvirus may occur, (ii) infecting cells with capripoxvirus and introducing the said construct into the said cells, (iii) screening the infected cells for recombinant capripoxvirus, (iv) isolating the recombinants from step (iii) and (v) growing the isolated viruses in a suitable host.

The construct may comprise a promoter for the heterologous gene, which will be any promoter which is functional in the cytoplasm of the capripoxvirus-infected cells. Thus, the promoter may interact with a polymerase which is also heterologous to both the capripoxvirus and the infected cell and which is encoded by another heterologous gene which is inserted in the recombinant capripoxvirus, as with the bacterial T7 system. Suitably, the promoter is derived from capripoxvirus itself but it may alternatively be derived from vaccinia virus, for

example it may be the promoter for the P7.5 protein (Mackett et al (1984), J. Virol. 49, 857-864; and Mackett & Smith, loc. cit.). Alternatively, the construct may not comprise a promoter at all but is so constructed as to recombine with the capripoxvirus at a site close to, and in correct reading frame with, an endogenous capripoxvirus promoter.

The flanking sequences may be synthesised by reference to the sequence of the insertion region or may be obtained from such a region and cloned. Typically, a length of about 100 nucleotides for each flanking sequence is sufficient to ensure recombination, but 120 nucleotides or more may be used.

In step (ii), the infection of the cells with the capripoxvirus may be before, after or at the same time as introduction of the DNA construct. The cells may be any which are permissive for capripoxvirus, such as most ovine, bovine and caprine derived cells, for example lamb testis, bovine palate, or ovine kidney cells. The DNA construct is preferably provided as a plasmid. The plasmid may be based on commercially available bacterial plasmids, such as pUC9, which may be cloned in bacteria and which have suitable markers for selection, for

example Amp^r. Transfection of the capripoxvirus-susceptible cells with the plasmid may be facilitated by known techniques involving calcium phosphate or electroporation.

The plasmid or other transfection vehicle is so chosen as to be non-infectious. Following introduction of the transfection vehicle into the host cell in step (ii), the vehicle is not propagated between cells. Hence, screening in step (iii) for the product of the heterologous gene effectively screens for recombinant viruses. Such screening may, for example, comprise an immunological assay for the antigen expressed by that gene or nucleotide hybridisation using sequences homologous to the heterologous gene. In one screening approach, the heterologous gene is in the first instance the E.coli beta-galactosidase gene (lac Z), thus allowing screening in step (iii) by the well-known technique using "X-gal" (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) in an agar overlay on the cell culture. Blue plaques are formed by recombinants which express the lacZ gene. Insertion of a further heterologous gene into the recombinant lac Z region, with concomitant deletion of the beta-galactosidase function, then allows selection by isolating colourless plaques against the parental blue

plaques. This approach may be useful where a suitable assay for the product of the second heterologous gene is not available.

Step (iv) will usually involve successive rounds of purification from plaques or cell-foci of infection, or end-point dilutions, and screening for recombinants in known ways. Amplification may be desirable during such procedures.

In step (v) the host may be any suitable cell line or, less preferably, any suitable living animal. Lamb testis and the other cell lines mentioned above are suitable.

The recombinant viruses may then be used to make vaccines in known ways, for example as described in Kitching, loc.cit. The virus is preferably included in the vaccine in an attenuated form, rather than killed.

The vaccines may be used to prevent the particular disease or diseases associated with the pathogen(s) of which an antigen or antigens are encoded by the virus of the invention, and may be used in sheep, goats, cattle (Bos bovis and Bos indicus), buffalo, llamas, alpacas and

vicunas. Generally speaking, the vaccines will be most suitable for use in areas where capripoxvirus-associated diseases are endemic and/or where the animals have all been vaccinated against capripoxvirus.

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The vaccines may be administered by any suitable route, including intra-nasally (as a spray), orally (for example in the feed or drinking water), intra-muscularly, sub-cutaneously, intra-venously or intra-dermally. However, experience with existing capripoxvirus vaccines shows that the intra-dermal route is not preferred and that the sub-cutaneous or intra-muscular routes are preferred.

For at least some time, typically 10 days, after the vaccine is administered to the animal, especially when the virus is attenuated rather than killed, the virus will be detectable in the animal's tissues, thus enabling an animal which has been vaccinated in accordance with the invention to be identified. The viral vectors of the invention may further include markers to aid such identification, if desired. In addition, the immunity of an animal which has been vaccinated in accordance with the invention may be distinguished from that of an animal which has been exposed to the corresponding pathogen since the former will include antibodies only to the particular antigen expressed by the modified viral genome and not to other antigens of the pathogen.

The invention will now be illustrated by way of the following non-limiting examples, with reference to the accompanying drawings, in which:-

Figure 1 shows the distribution of HindIII restriction enzyme recognition sites on the genome of capripoxvirus strain KS-1, together with a scale representing length in numbers of kilobase pairs;

Figure 2 shows the nucleotide sequence of the S fragment identified in Figure 1, with the region labelled as CF8 corresponding to the thymidine kinase (TK) region;

Figure 3 shows the sequence from the terminal region of the genome of capripoxvirus isolate KS-1, reading in a telomere-proximal telomere-distal direction. The terminal HindIII site (AAGCTT) of the genome occurs in the sequence. This site, and the Sau3A1 (GATC) site defining the proximal end of clone pPG64, are underlined and emboldened; and

Figure 4 shows the distribution of stop-codons in the 6 possible reading frames for the sequence between approximately 700 and 2,400 bp from the apex of the terminal hairpin loops of capripoxvirus strain KS-1 DNA, the telomere proximal and distal ends bring on the left and right, respectively.

EXAMPLE 1: Insertion into the TK region

The thymidine kinase (TK) gene of capripoxvirus was localized on the genome of isolate KS-1 (previously referred to as 'Kenya Sheep and Goat Pox') as described

in reference 1. The gene was found to be present within HindIII fragment S (as shown on Figure 1), and HindIII S was completely sequenced.

To insert foreign DNA into the TK gene of capripox-virus, we used a variation of the previously described method (Mackett & Smith, loc. cit.) of: cloning a construct comprising (i) the genomic region containing the candidate insertion site in a bacterial plasmid and (ii) a foreign gene attached to a poxvirus promoter at the site in the plasmid clone equivalent to the intended insertion site in the virus genome; and transfecting the recombinant plasmid into poxvirus infected cells.

In the experiments performed here, the genomic region containing the intended insertion site was the KS-1 genomic DNA fragment HindIII S, which was cloned in the plasmid vector pUC9. The plasmid was linearized by incubating with the restriction enzyme KpnI, for which a single site is present in the clone, and is located within the KS-1 TK gene.

The bacterial lacZ gene coupled to the vaccinia virus promoter P7.5 (P7.5-lacZ), was inserted at the KpnI site in the above plasmid using T4 ligase, after cleaving

P7.5-lacZ from another plasmid with the restriction enzyme BamHI, and treating both the BamHI and the KpnI fragments with Klenow enzyme. The resulting plasmid was named pPG55.

Lamb testis (LT) cell cultures infected with capripoxvirus isolate KS-1 were transfected with the pPG55, and the cultures were incubated at 37°C for 5 days. Further LT cell cultures were infected with the harvested virus and after seven days incubation these cultures were overlaid with low gelling temperature (LGT) agarose containing the substrate X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) ('X-gal overlay'). After a further 24 hours blue foci were visualised through the X-gal overlay, and virus was picked through the agarose and subjected to further rounds of plaque purification.

EXAMPLE 2: Identification of Further Insertional Sites

A library of capripoxvirus KS-1 DNA in plasmid pPG2 was produced by partial digestion of the capripoxvirus DNA with Sau3A, and cloning of the resulting fragments into BamHI linearised pPG2. pPG2 was derived from the commercially available pUC9 by removal of the multiple cloning site by digestion with HindIII and EcoR1, filling

in of the single-stranded ends with Klenow enzyme, and ligation (using T4 ligase) of a synthetic BamHI linker (GGGATCCC) to recircularise the plasmid DNA. The ligation mixture was used to transform E.coli strain TG1 and the bacteria plated onto LB agar containing ampicillin. Individual bacterial colonies were picked at random and their plasmid DNAs prepared. Plasmid DNAs containing HindIII sites were identified by digestion with HindIII and analysis by agarose gel electrophoresis. 40 clones carrying HindIII sites located at different positions on the capripoxvirus genome were digested with HindIII and the HindIII P7.5-lacZ fragment was inserted into each using T4 ligase to create a series of trial insertional vectors for at least 10 different sites in the capripoxvirus genome. Following cloning in E.coli and preparation of purified plasmid DNA, each trial insertional vector was transfected into capripoxvirus KS-1 infected lamb testis cells and the presence of blue plaques/foci was screened for. The presence of blue plaques/foci identified those insertional vectors carrying sequences which directed insertion by recombination at sites which are not essential for capripoxvirus replication and infection in lamb testis cells. In this way we have identified three HindIII sites within non-essential regions of capripox-virus DNA into which heterologous DNA

can be inserted.

This represents a general method for identifying non-essential insertion sites in the capripoxvirus genome. Other restriction enzymes may be used instead of HindIII, preferably those which (for the capripoxvirus genome) cut at about the same frequency, namely about 25-35 times, preferably about 30 times. Infrequent cutting results in insertion sites being missed, whereas too frequent cutting yields fragments which are too small to be practicable. Generally, one would use enzymes which recognise a sequence of 5 to 7 nucleotides, preferably 6. However, as the capripoxvirus genome is rich in A's and T's, an enzyme recognising a 4-nucleotide sequence rich in G's or C's might also be suitable.

Sau3A is a suitable enzyme for the initial digestion as it recognises frequently occurring sites (about 600, i.e. an average distribution of about one per 250 b.p.); a partial, rather than complete, digest then yields virtually random, conveniently-sized fragments likely to contain a HindIII site. Other enzymes, such as HaeIII, may be used for differing frequencies of occurrence of

the recognition sites. The partial digests may be pooled. The pool itself may be cloned or one may select a particular size class of fragment for cloning.

EXAMPLE 3: Insertion into the CT4 Region

The work outlined in this Example is based on clones covering a region within the terminal repeats of the genome of capripoxvirus isolate KS-1. This region covers two open reading frames (ORFs) named CT3C and CT4. Figure 3 shows the 1651 b.p. nucleotide sequence of the above mentioned region. Figure 4 shows the positions of the stop codons in the sequence. ORFs CT3C and CT4 are marked; CT3C lies between nucleotide 252 and 647 and CT4 lies between nucleotide 839 and 1621 in this sequence.

We constructed a plasmid, pPG64, to enable P7.5-lacZ to be inserted within CT3C; more specifically at the Hind III site between nucleotides 561 and 566 of the sequence shown in Figure 3. This HindIII site divides HindIII fragments W1 and 11 at the left-hand end of the genome and fragments F and W2 at the right-hand end (see Figure 1). Clone pPG64 was made by ligating a Sau3A partial digest fragment of KS-1 DNA of size 1648 b.p., starting at nucleotide 440 on the sequence shown in Figure 3, in a

derivative of the commercially available plasmid cloning vehicle pUC9, named pPG2 (see Example 2). The resulting plasmid, in which P7.5-lacZ is cloned at the HindIII site of pPG64, is called pPG65.

Recombinant capripoxvirus KS-1 was generated using pPG65 by the method described in the last paragraph of Example 1. In the experiments using pPG65, virus obtained from blue viral foci was propagated on LT cells, and gave a further round of blue foci. These in turn were propagated by several additional rounds of plaque purification and shown to maintain the ability to express blue foci.

The capripoxvirus isolates used in the work described herein are typical of wild-type capripoxvirus and the techniques described and claimed herein do not depend upon the particular strain or isolate which is used. Other isolates may have minor variations in their genomes, but it will be well within the capabilities of the man skilled in the art to adapt the techniques to such other isolates. For example, it is believed that the region of another isolate (InS; Gershon & Black (1987) Virol. 160, 473-476) which corresponds to the ORF in KS-1

labelled herein as CT4 is a non-coding region. Such a region would clearly be a suitable candidate for insertion of the heterologous gene.

CLAIMS

1. A capripoxvirus capable of infecting and replicating in capripoxvirus-susceptible cells and carrying at least one heterologous gene encoding an antigen of a pathogen such that the gene will be expressed during transcription and translation from the capripoxvirus genome in the said cell.

2. A capripoxvirus according to Claim 1 wherein at least one said heterologous gene is inserted in one of the following regions of the capripoxvirus genome:

(a) the CT3C region,

(b) the thymidine kinase region,

(c) the CT4 region, or

(d) the Q2 region

the regions being as defined herein.

3. A capripoxvirus according to Claim 1 or Claim 2 wherein the antigen is associated with bluetongue disease, ephemeral fever, foot-and-mouth disease, mucosal disease, border disease, rabies, Rift Valley fever, rinderpest, peste des petits ruminants, vesicular stomatitis, Nairobi Sheep disease, lentivirus infections, trypanosomiasis, theileriosis, clostridial infections, tuberculosis, haemonchiosis, schistosomiasis or Brucella infections.

4. A vaccine comprising a capripoxvirus according to any one of the preceding claims and a carrier and/or adjuvant.

5. A vaccine according to Claim 4 wherein the vaccine is suitable for sub-cutaneous delivery.

6. A process for preparing a capripoxvirus according to any one of Claims 1 to 3 by (i) constructing a DNA construct comprising the heterologous gene and two sequences which flank the heterologous gene and which are homologous to respective parts of the capripoxvirus DNA such that recombination of the construct with capripoxvirus may occur and (ii) infecting cells with capripoxvirus and introducing the said construct into the said

cells, (iii) screening the infected cells for recombinant capripoxvirus, (iv) isolating the recombinants from step (iii) and (v) growing the isolated viruses in a suitable host.

7. A process according to Claim 6 wherein the construct comprises a promoter for the heterologous gene, the promoter being derived from capripoxvirus.

8. A method of treating or preventing pathogen-caused disease in cattle, buffalo, sheep or goats comprising administering to the cattle, buffalo, sheep or goats a non-toxic disease-protective amount of vaccine according to Claim 4 or 5.

9. A cow, buffalo, sheep or goat treated by the method of Claim 8.

10. A method of identifying non-essential insertion sites in a capripoxvirus genome, comprising (i) preparing a library of capripoxvirus DNA in a suitable plasmid or other vector, (ii) transforming a culture of a suitable host with at least part of the said library, (iii) plating out the transformed host cells to give individual colonies, (iv) isolating plasmid DNA from the said

individual colonies, (v) digesting the said DNA with a suitable restriction enzyme, (vi) forming a construct by inserting into the insertion site thus formed a heterologous gene encoding a marker protein, (vii) transfecting host cells with the said construct, and (viii) screening for host cells which (a) are infected with live poxvirus and (b) express the marker protein.

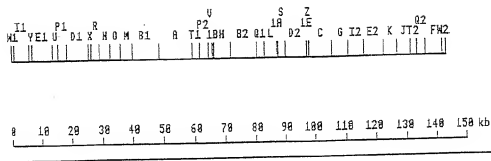
11. A method according to Claim 10 wherein step (i) includes partial digestion with Sau3A.

12. A method according to Claim 10 or 11 wherein the restriction enzyme of step (v) recognises 25-35 sites on the poxvirus genome.

13. A method according to Claim 12 wherein the restriction enzyme of step (v) is HindIII.

14. A method according to any one of Claims 10 to 13 wherein the marker protein is beta-galactosidase.

1/4

Figure 1

3/4

GATCATTTCCAAATACAAGTGAGGCATCCTTTTTGAAAGATTCAAAAACCTA
AGAACACCTTTCCAGCAACCTCCCTGGACGAAAATGCCAGTGATGACCAA
AACAAATATCAGAGATGGCGGTTGTGATATCATCATCTGAAAAAGTT
GTTTCGGTAGACATAGTTGCCGGAAGACATCTTGTAATAATGAACGGGAAA
AAAGTCTCTTTTTAACCCGAACTTGTTATTTCCGTGTCTTATTTTTCAAAA
TTATTCAACCATATTATCATCCTTTCAACTAAT-CTTTTCTAGGTGGTT
CCTTTATTGTGTTTTGATTACAGTAGTATTCGTAGTGTTTTTAAATGGA
TTAGGAATTTTAATAAAATCAAAAACCTGCATTATCATTTC-AAATTTTAT
ATCGCTTGATCTTTTTTATCTAATACAGTTAATGGTAGATCATTATTTA
TTTTCTTACTTTTCTATAGGTTTGTGATTAGTGTTTATATTGTCTTGGATT
TTTTCTATCCTTATCCAAGACAGAATCGAACGGATTTAGGTTTCCAAACAT
GAAGGAGAT AAGCTTTTGCATTGGAAACATTAATAATGAATACAAAGTAT
ATATAATTAAATTACATAAATCTAGCTATATAAAAAATACACAACATACAA
ACCTTTAGCTAAGCTATTAAATGAGTAGTTGACAAAAATCCTTTTATTGCATA
TATCACAAATCCTAAATCTTTAGTATAAGAAGTAATGTCCCTCTTACAC
ATCTCCATATACTAAAGATTATTTTATGAATTAAATGCAAAAATCCACCAG
ACTAAAAAAGAACGGAAGAACTCCTAAGTTTCTTATTCAATAATTTATATAT
GTGAAAAGAACAAAAATAAATATTATATCGTCTTTTGTATAAACAATCCTA
TAGTTCATTATGATGAATATTTTTTAAATATAATTATGCTGATGTTTCAT
AATATTTTTTTTCTATCAAATTTCCCAACACTGCACGTTTTTAAATAAATTT
ACATGTTCACTAAATTTTACAGCTGAGCAATCTATTTTATTATTAAACCC
AAAAATCTTTTTTCTGTAGATATATATTTTTTTTTCACATTCACTTTCTAA
CTATAACACTTGAACCTTACATGTTTTACACAACCTAATATCTGTTGTGTATA
ACAAATTTCTAAATCATAATCTTTTAAACCTTACACTCTCAACGTATGATTT
CATTTCTGTAGTTACTTTGATCACAAAGAAATTGAC-ATGATATTTCTCTTA
GCACACTGTGGCAGCGAATACAGAGTGTTATATTACTGGTTGATATTTCC
ATATATACATATTTACCAATGCATAAACTGCTTTGTTAACTATCTAA
TATAAAGTTGTTTTGTGTTTCTTCTTTGGAACAATCATCATGTGGCGAAAA
TTTTAAATCCATTTCCCTCTGTTAGAAGTCTCCAGGTCCTTGAATTTTA
TAACAGCCAGTTTTAATCGCTCTAAAATTTTCTGTTTGGATACATAATCC
TACTTTCGATTTCCCATGTGTTATTTTCTTCTTC-GTGCATCTCTTTATTG
ATATT-CGCTTACACTACTAGTAAAAATGAATAGTTGCAAAAAGAAATG
AACAAAGTTAATGATGACATATTGG-TGGTTGTTTTGACGTATTGACGAC

Figure 3

4/4

Sequence length = 1649 nt
Full scale = 1649 nt
Left end inset = 0 nt

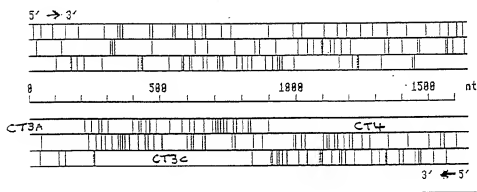


Figure 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00965

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *	
According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : C 12 N 15/86, A 61 K 39/275, //C 12 N 15/30, C 12 N 15/31, A 61 K 39/002, A 61 K 39/02, A 61 K 39/12, A 61 K 39/295, ./.	
II. FIELDS SEARCHED	
Minimum Documentation Searched *	
Classification System	Classification Symbols
IPC ⁵	C 12 N
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *	
III. DOCUMENTS CONSIDERED TO BE RELEVANT *	
Category *	Relevant to Claim No. 12
Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	
X Annual Report of the Institute for Animal Health of the Agricultural and Food Research Council, 1987, Institute for Animal Health (Compton, Newbury, GB), D.N. Black: "Virus vectors (P)", page 58, see page 58, column 1, line 24 - column 2, line 29 cited in the application --	1,4-6
A J. Gen. Virol., vol. 67, 1986 (GB), D.B. Boyle et al.: "Identification and cloning of the fowlpox virus thymidine kinase gene using vaccinia virus", pages 1591-1600, see the whole article --	1
A Gene, vol. 47, no. 2/3, 1986, Elsevier Science Publishers B.V. (Biomedical Division), (Amsterdam, NL), D. Panicali et al.: "Vaccinia virus ./.	10
<p>* Special categories of cited documents: ¹³</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>	
IV. CERTIFICATION	
Date of the Actual Completion of the International Search 23rd November 1989	Date of Mailing of this International Search Report 28 DEC 1989
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer L. ROSSI

INTERNATIONAL SEARCH REPORT PCT/GB 89/00965

International Application No -2-

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : (C 12 N 15/00; C 12 R 1:91)		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁵		
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
	vectors utilizing the beta-galactosidase assay for rapid selection of recombinant viruses and measurement of gene expression", pages 193-199, see the whole article --	
A	Virology, vol. 164, 1988, Academic Press Inc., P.D. Gershon et al.: "A comparison of the genomes of capripoxvirus isolates of sheep, goats, and cattle", pages 341-349 --	
A	Virology, vol. 160, 1987, Academic Press Inc., P.D. Gershon et al.: "Physical characterization of the genome of a cattle isolate of capripoxvirus", pages 473-476 --	
A	Trends in Biotechnology, vol. 6, no. 2, ./.	
<p>* Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (see specification)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
	28 DEC 1989	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	L. ROSSI	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
	<p>February 1988, Elsevier Publications (Cambridge, GB), J. Tartaglia et al.: "Recombinant vaccinia virus vaccines", pages 43-46</p> <p>--</p>	
A	<p>J. Gen. Virol., vol. 67, no. 10, 1986, SGM (GB), M. Mackett et al.: "Vaccinia virus expression vectors", pages 2067-2082 cited in the application</p> <p>--</p>	
P,A	<p>J. Gen. Virol., vol. 70, 1989, SGM (GB), P.D. Gershon et al.: "The nucleotide sequence around the capripoxvirus thymidine kinase gene reveals a gene shared specifically with leporipoxvirus", pages 525-533</p> <p>----</p>	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 8..... because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.